EMBO
Molecular Medicine

Onco-miR-155 targets SHIP1 to promote TNFα-dependent growth of B cell lymphomas

*Irene M. Pedersen[1], Dennis Otero[1], Elaine Kao[1], Ana V. Miletic[2], Christoffer Hother[3], Elisabeth Ralfkiaer[4], Robert C. Rickert[2], Kirsten Gronbaek[3], Michael David[1,5]**

Keywords: inflammation; lymphoma; microRNA; phosphatase; TNFα

DOI 10.1002/emmm.200900028

Received April 21, 2009
Revised May 18, 2009
Accepted May 21, 2009

Non-coding microRNAs (miRs) are a vital component of post-transcriptional modulation of protein expression and, like coding mRNAs harbour oncogenic properties. However, the mechanisms governing miR expression and the identity of the affected transcripts remain poorly understood. Here we identify the inositol phosphatase SHIP1 as a bonafide target of the oncogenic miR-155. We demonstrate that in diffuse large B cell lymphoma (DLBCL) elevated levels of miR-155, and consequent diminished SHIP1 expression are the result of autocrine stimulation by the pro-inflammatory cytokine tumour necrosis factor α (TNFα). Anti-TNFα regimen such as eternacept or infliximab were sufficient to reduce miR-155 levels and restored SHIP1 expression in DLBCL cells with an accompanying reduction in cell proliferation. Furthermore, we observed a substantial decrease in tumour burden in DLBCL xenografts in response to eternacept. These findings strongly support the concept that cytokine-regulated miRs can function as a crucial link between inflammation and cancer, and illustrate the feasibility of anti-TNFα therapy as a novel and immediately accessible (co)treatment for DLBCL.

INTRODUCTION

B cell survival and fate determination are strongly dependent upon phosphatidylinositol 3-kinase (PI3K) signalling (Alizadeh et al, 2000; Fruman, 2004). PI3K catalyses the conversion of membrane phosphatidylinositol-(4,5)-bisphosphate to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3), which acts as a second messenger to recruit pleckstrin-homology domain containing adapters and kinases such as 3'-phosphoinositide-dependent kinase (PDK), AKT, phospholipase C γ2 (PLCγ2), Bruton's tyrosine kinase (BTK), downstream of kinase (DOK) and others. Subsequent activation/inactivation of additional effectors including serum and glucocorticoid-inducible kinase (SGK), target of rapamycin (TOR), protein phosphatase 2A (PP2A), forkhead box O (FoxO) and Cyclin D/E mediates diverse biological responses such as survival, proliferation, migration, adhesion and differentiation. PI3K signals are antagonized by two lipid phosphatases: the 3′-inositol phosphatase (PTEN) and the 5′-inositol phosphatase (SHIP). It was recently discovered that in contrast to T cells, B cells do not undergo malignant transformation upon *PTEN* deletion (AV Miletic, A Mills, D Mills, IM Pedersen, H Morse, J Ravetch, S Bolland, RC Rickert, unpublished work). Similarly, deletion of *SHIP1* in B cells is insufficient to generate B cell lymphoma. However, concomitant ablation of both *SHIP1* and *PTEN* in murine B cells induces lethal lymphoma resembling DLBCL with 100% penetrance, revealing a novel role for SHIP1 as a tumour-suppressor (Miletic et al, unpublished work).

Disease in B cell non-Hodgkin lymphoma (B-NHL) patients is graded according to the International Prognostic Index (IPI), which (among other things) assigns low scores for the involvement of single lymph nodes, intermediate scores for progression to multiple lymph node involvement and high scores for systemic nodal and non-lymphoid metastases.

(1) Section of Molecular Biology, Division of Biological Sciences, University of California San Diego, La Jolla, CA, USA.
(2) Burnham Institute for Medical Research, La Jolla, CA, USA.
(3) Department of Hematology, Copenhagen University Hospital, Copenhagen, Denmark.
(4) Department of Pathology, Copenhagen University Hospital, Copenhagen, Denmark.
(5) Moores Cancer Center, University of California San Diego, La Jolla, CA, USA.
***Corresponding author:** Tel: +1 858 8221108; Fax: +1 858 8221106;
E-mail: midavid@ucsd.edu

Significant effort towards understanding disease progression in human patients has suggested that B-NHL, in addition to multiple cancer-promoting genetic 'hits', is fuelled by non-specific stimuli such as B cell antigen receptor (BCR)-mediated recognition of undefined self-antigens or soluble survival factors. In support of this latter idea, B-NHL tumours often display several hallmarks of antigen-mediated clonal selection or cytokine dependence. DLBCL is clinically, morphologically and genetically a heterogeneous group of malignant proliferation of large lymphoid B cells that accounts for approximately 40% (25,000 cases/year) of adult NHLs (Coiffier, 2001). Standard chemotherapy has been recently expanded from CHOP (Cyclophosphamide/Doxorubicin/Vincristine/Prednisolone) to R-CHOP with the inclusion of Rituximab, an anti-CD20 monoclonal antibody, which improved treatment success to an overall 3-year relapse free survival of DLBCL patients at 53–63.1% (Habermann et al, 2006; Pfreundschuh et al, 2008). Two prognostically different subgroups of DLBCL have been identified with distinct gene expression profiles either characteristic of normal germinal centre (GC) B cells or of activated memory B cells. The GC B-cell-like subgroup was correlated with a significantly better prognosis (5-year survival: 76%) in comparison to the activated B cell-like (ABC or non-GC) subgroup (5-year survival: 16%) (Alizadeh et al, 2000; Shipp et al, 2002). Furthermore, previous work by Rai et al and a very recent report published during the preparation of this manuscript revealed unique miR signatures for the two classes of DLBCL (Malumbres et al, 2009; Rai et al, 2008).

In the present study we show elevated miR-155 levels and concomitant suppression of SHIP1 expression in non-GC compared to the GC-DLBCL cell lines or primary tumour samples. Furthermore, we demonstrate that augmented miR-155 expression in DLBCL is a consequence of increased TNFα production rather than genomic mutation, and can therefore be reversed *in vitro* and *in vivo* through neutralization of TNFα, with subsequent inhibition of cell proliferation and tumour growth.

RESULTS

To determine whether a correlation exists between survival of patients with B cell malignancies and expression levels of PTEN and SHIP, we utilized ONCOMINE to query published cDNA array results. Analysis of data originating from gene expression studies by Alizadeh and Rosenwald (Alizadeh et al, 2000; Rosenwald et al, 2002) revealed that SHIP1 levels are significantly decreased in DLBCL compared to more indolent chronic lymphocytic leukaemia (CLL) or follicular lymphoma (FL) (Fig 1A). Strikingly, SHIP1 levels within the non-GC subset displayed strong correlation with overall survival (Fig 1B) suggesting that SHIP1 expression levels are useful prognostic indicators of survival among DLBCL patients.

In order to determine the molecular mechanisms that mediate the observed decrease in SHIP1 expression in DLBCL, we determined the methylation status of the SHIP1 promoter in 44 non-GC and GC-DLBCL specimens, as well as screened for mutations in the coding regions and splice sequences, but did not detect any differences among the samples (not shown). We next considered the possibility that post-transcriptional regulatory events mediated by small non-coding RNAs might alter the expression of SHIP1 in these haematopoietic malignancies. Indeed, in addition to differential expression of coding genes, non-GC-type lymphoma cells express elevated levels of several miRs. Scanning of the SHIP1 3′-untranslated region (3′-UTR) revealed perfect sequence complementarity with the seed sequence of miR-155 (Fig 1C), an onco-miR whose ectopic expression gives rise to B cell malignancies (Costinean et al, 2006), but whose cellular targets have remained elusive.

Using a prototypic cell line model of the GC- and a non-GC-subtypes of DLBCL, Lawrie et al showed that in addition to miR-155, miR-221 and miR-21 were also over-expressed in non-GC-type but not GC-type lymphoma cells (Lawrie et al, 2007). To determine miR-155 and SHIP-mRNA levels in primary DLBCL patient samples without concern of potential contamination by non-malignant tissue, we isolated tumour cells by laser-capture-microdissection (LCD) from frozen lymph node biopsies. As shown in Fig 1D, miR-155 expression was significantly higher in non-GC-DLBCL compared to GC-DLBCL, whereas SHIP1 mRNA levels were lower in non-GC-DLBCL, consistent with the notion of attenuation of SHIP1 expression by miR-155.

To establish a link between miR-155 and SHIP1 expression beyond mere correlation, the non-GC-DLBCL cell line OCILY-3 was transfected either with a non-specific control-miR, miR-1 or miR-155. Neither miR-1 nor the control miR affected SHIP1 mRNA levels, whereas introduction of miR-155 resulted in a clear decrease in SHIP1 mRNA (Fig 2A). More importantly, neutralization of endogenous miR-155 by means of a synthetic anti-miR resulted in a dramatic increase in SHIP1 mRNA compared to a transfection of a non-specific anti-miR control (Fig 2A). As anticipated, the modulation of SHIP1 mRNA levels by miR-155 or anti-miR-155 is reflected by accompanying changes in SHIP1 protein expression (Fig 2B, left panel), and is not unique to OCILY-3 cells, but is also observed in the OCILY-10 and Toledo cells, a widely used cell line model representative of non-GC-DLBCL (Fig 2B, middle and right panel). In contrast, miR-155 did not alter the expression of hSHIP2 protein (Fig S1 of Supporting Information).

Lastly, analysing SHIP1 protein levels in B-cells isolated from WT and miR-155 transgenic (tg) mice, we found that SHIP1 is greatly reduced in miR-155 (tg) B-cells as compared to their WT counterparts (not shown), further supporting the notion that miR-155 post-transcriptionally regulates SHIP1 expression.

To investigate whether miR-155 targets and represses SHIP1 directly through 3′-UTR interaction, we inserted the 3′-UTR of hSHIP1 into a reporter plasmid at the 3′ end of luciferase mRNA driven by a CMV promoter (WT 3′-UTR-luc) and analysed the effect of miR-155 on luciferase expression. As anticipated, co-transfection of miR-155 attenuated expression of luciferase from the WT 3′-UTR-luc reporter (Fig 2C, bars 1 and 3), whereas no inhibition was observed when a control miR (miR-CTL) was used (Fig 2C, bar 2). Similarly, a mutated miR-155 (miR-155mut) whose seed sequence had been altered, failed to suppress the luciferase activity originating from WT 3′-UTR-luc

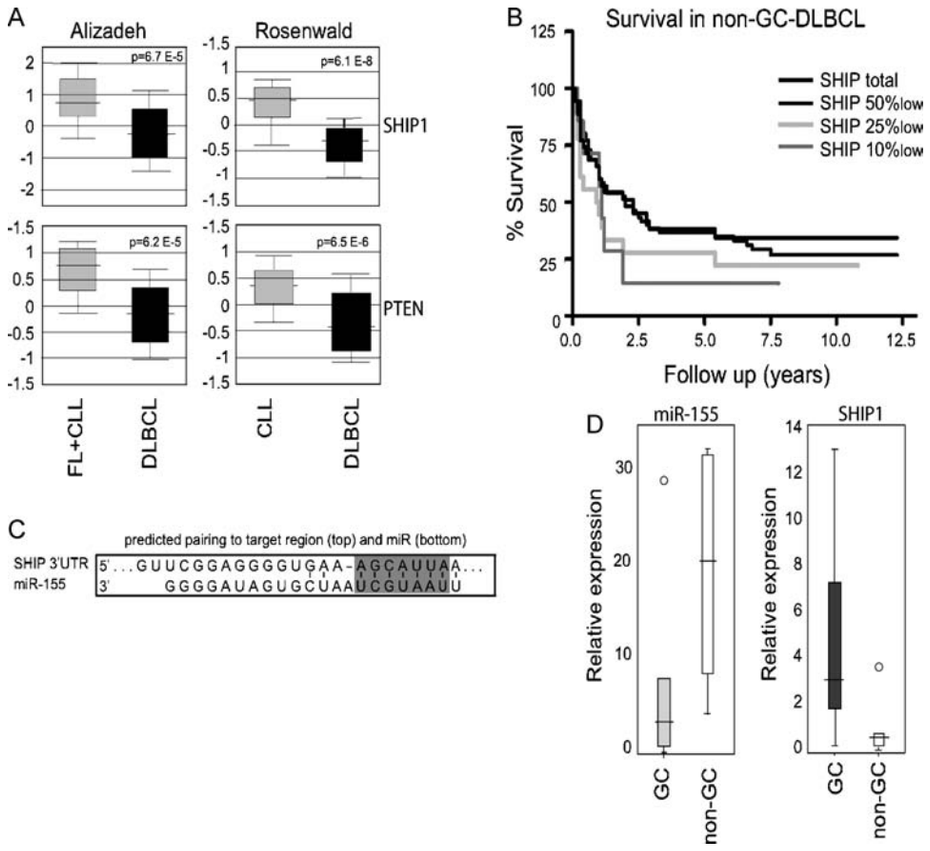

Figure 1. Differential SHIP1 expression correlates with prognosis of DLBCL.

A. Two independent cDNA arrays (Alizadeh (Alizadeh et al, 2000) and Rosenwald (Rosenwald et al, 2002)) were analysed in Oncomine (www.oncomine.org) for expression of SHIP1 in DLBCL, CLL and FL. Y-axis represents normalized expression values (mean ± standard deviation; *p*-values are derived from Student's *t*-test).

B. Kaplan–Meier plots (Oncomine; Rosenwald et al, 2002) showing overall survival of 72 non-GC-DLBCL patients using defined cut-off values for SHIP1 expression (patient sub-groups defined as having the lowest 50% (50% low: 34 patients), lowest 25% (25% low: 18 patients) or lowest 10% (10% low: 7 patients) relative SHIP1 expression).

C. Sequence alignment of the SHIP1 3′-UTR and miR-155 (TargetScan; seed sequence is highlighted in grey).

D. Frozen lymph node sections of ABC (non-GC) and GC-DLBCL patients were stained with H&E, and tumour cells (>1,000 cells/specimen) were isolated by LCD. RNA was analysed for miR-155, SHIP1, U6 and GAPDH expression. Data represent 5 (GC) and 6 (non-GC) specimens, respectively (Wilcoxon rank sum test; *p*-value miR-155: 0.08225; *p*-value SHIP1: 0.08125).

plasmid (Fig 2C, bar 4). To further demonstrate the specificity of this interaction, we also generated a luciferase reporter in which the hSHIP1 3′-UTR was modified to be complementary to the seed sequence of miR-155mut (Mut 3′-UTR-luc). This reporter system was resistant to the inhibitory effects of miR-155 (Fig 2C, bar 7), but was repressed by miR-155mut (Fig 2C, bar 8). These results demonstrate that miR-155 inhibits SHIP1 expression by directly interacting with the hSHIP1 3′-UTR.

miR-155 is one of only a few miRs whose expression has been shown to be regulated by extra-cellular ligands. O'Connell et al demonstrated that miR-155 expression can be induced in macrophages by stimulation with lipopolysaccharide (LPS) or TNFα (O'Connell et al, 2007), and analysis of the activation pathway indicated that miR-155 is an AP1-responsive transcript involving the JNK pathway. B cells not only produce TNFα (Endres et al, 1999; Pasparakis et al, 1996), but they upregulate miR-155 in response to this cytokine in a JNK-dependent manner similar to macrophages (Fig S2 of Supporting Information). As previous studies showed that patients with non-GC-DLBCL display elevated serum TNFα levels compared to those suffering from GC-DLBCL or other B cell malignancies (Pedersen et al, 2005), the possibility that non-GC-DLBCL cells create an auto-stimulatory loop leading to elevated miR-155 levels by producing TNFα appeared intriguing.

To test this hypothesis, three non-GC-DLBCL cell lines (OCILY-3, OCILY-10, Toledo) were cultured in the presence of eternacept (Enbrel®), an antagonistic soluble TNFα receptor that finds widespread clinical use in the treatment of inflammatory diseases such as rheumatoid arthritis and Crohn's disease. Strikingly, this anti-TNFα regimen led to a substantial

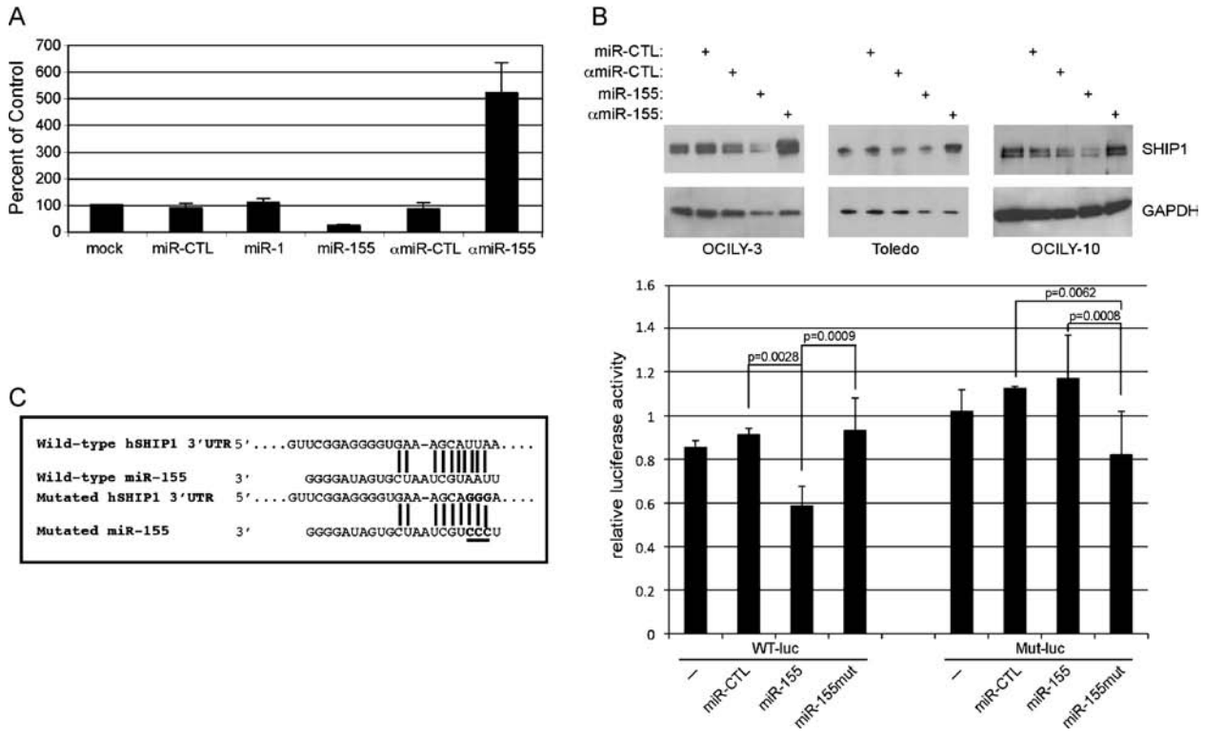

Figure 2. miR-155 attenuates SHIP1 expression. Non-GC-DLBCL cell lines OCILY-3, OLILY-10 and Toledo were transfected with 50 μM of either non-specific miR (miR-CTL), miR-1, miR-155, non-specific anti-miR (αmiR-CTL) or anti-miR-155 (αmiR-155).

A. Cells were harvested 12 h post transfection, SHIP1 mRNA levels were determined by qPCR and normalized to GAPDH (mean ± SD; $n = 4$).

B. Same as (A), except cells were lysed after 72 h, and Western blot analysis was performed using antibodies against human SHIP1 and GAPDH.

C. Luciferase constructs containing either the wild-type (WT) or mutated (Mut) hSHIP1 3′UTR (50 ng) and the indicated miRs (1 nM; miR-CTL, miR-155 or mutated miR-155 (miR-155mut)) were transfected into 293T cells, and luciferase activity was determined 24 h post transfection. Graph indicates relative luciferase values after normalization to co-transfected renilla luciferase (mean ± SD, $n = 5$, p-values derived from paired t-test). WT and mutant SHIP1 3′-UTRs as well as WT and mutant miR-155 are illustrated.

reduction in miR-155 expression in all three cell lines, but did not occur in the GC-DLBCL cell line SUDHL-4 (Fig 3A). Similar results were obtained when infliximab (Remicade®), a neutralizing humanized monoclonal antibody against TNFα was used (not shown). Importantly, reduced miR-155 levels were accompanied by a concomitant increase in SHIP1 protein levels in the non-GC-DLBCL cells (Fig 3B).

The biological significance of these observations is evidenced by the finding that eternacept imposes significant antiproliferative effects upon the three non-GC-DLBCL cell lines, but did not produce any growth modulation in the GC-DLBCL cells, or in Daudi Burkitt lymphoma (Fig 3C).

Our results thus far demonstrated that in non-GC-DLBCL, elevated levels of miR-155, and consequent abrogation of SHIP1 expression, are mediated through autocrine stimulation of cells by TNFα, a proinflammatory cytokine whose serum levels are known to be elevated in DLBCL patients (Pedersen et al, 2005). To explore the potential efficacy of anti-TNFα regimen as a treatment for non-GC-DLBCL patients, we employed xenograft models in which non-obese diabetic/severe combined immunodeficiency (Nod/SCID) mice were subcutaneously inoculated with non-GC-DLBCL Toledo cells. Upon establishment of palpable tumours, the animals received either 100 μg eternacept or solvent intravenously every three days, and tumour size was measured after 2, 4 or 6 days. As shown in Fig 4A, eternacept treatment resulted in a slight, but detectable inhibition in tumour growth at day 4, and produced a substantial reduction in tumour burden after 6 days. In concurrence, the analysis of the excised xenograft tumours revealed increased SHIP1 protein levels in tumours from eternacept-treated mice compared to tumours isolated from animals that received phosphate buffered saline (PBS) (Fig 4B).

DISCUSSION

Soon after the discovery of the first mammalian miR some 12 years ago, it became evident that this class of molecules plays a critical role in global gene regulation, and a likely impact on cellular survival and death pathways. It is not surprising that tremendous strides have been made in defining miRNAs expression profile in human cancers. High throughput analyses,

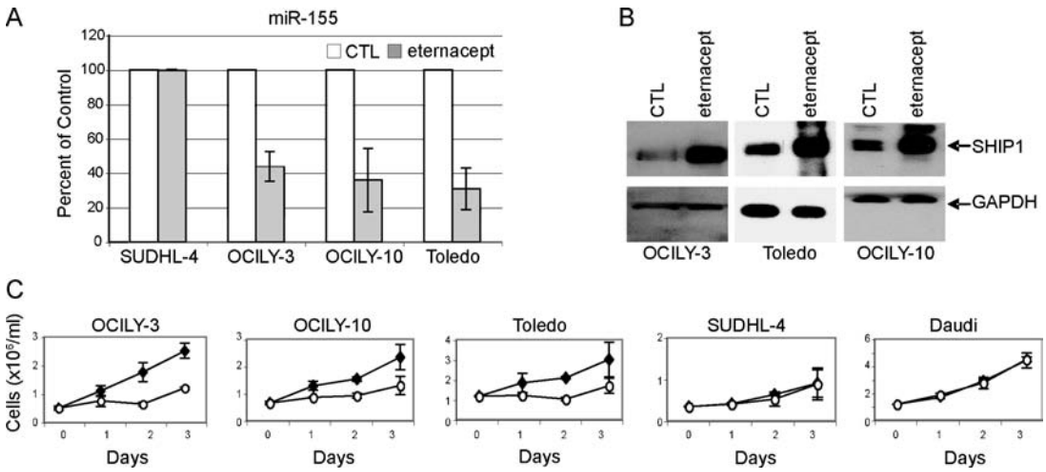

Figure 3. Autocrine stimulation of non-GC DLBCL by TNFα. Non-GC-DLBCL cell lines OCILY-3, OCILY-10, Toledo and the GC-DLBCL cell line SUDHL-4 and the Burkitt lymphoma Daudi were treated with 100 ng/ml eternacept as indicated.

A. miR-155 expression levels were determined by qPCR and normalized to U6 RNA (mean ± SD; $n = 3$);

B. Cells were harvested after 72 h, and SHIP1 protein expression was determined by Western blot analysis;

C. Cell proliferation was determined in triplicate cultures 24, 48 and 72 h after addition of eternacept (mean ± SD of at least three independent experiments).

utilizing various analytical methods demonstrated that miR expression was commonly dysregulated in a multitude of human cancers (Calin & Croce, 2006; Lu et al, 2005; Roldo et al, 2006). miR expression profiling has shown promise in defining malignant status in retrospective studies. It has even been suggested that microRNA expression profiling can distinguish cancers according to diagnosis and developmental stage of the tumour to a greater degree of accuracy than traditional gene expression analysis.

More than 50% of annotated human miRs genes are located in fragile chromosomal regions that are susceptible to amplification, deletion or translocation during the course of tumour development (Calin et al, 2004). Moreover, recent evidence indicates that some miRs function either as oncogenes or tumour suppressors (Esquela-Kerscher & Slack, 2006; He et al, 2005; Johnson et al, 2005). The first study documenting abnormalities in miR expression in tumour samples focused on CLL. Deletion of chromosome 13q14 is the most frequent chromosomal abnormality in this disorder. Croce and coworkers demonstrated that tumour suppressor activity is likely provided by two miRNAs, miR-15a and miR-16-1 (Calin et al, 2002). Cimmino et al identified a conserved site for miR-15a and miR-16-1 in the 3′-UTR of the *bcl-2* mRNA, which encodes the anti-apoptotic protein Bcl-2 (Cimmino et al, 2005).

miR-155 was the first onco-miR described, whose expression in B cells alone is sufficient to trigger malignant transformation,

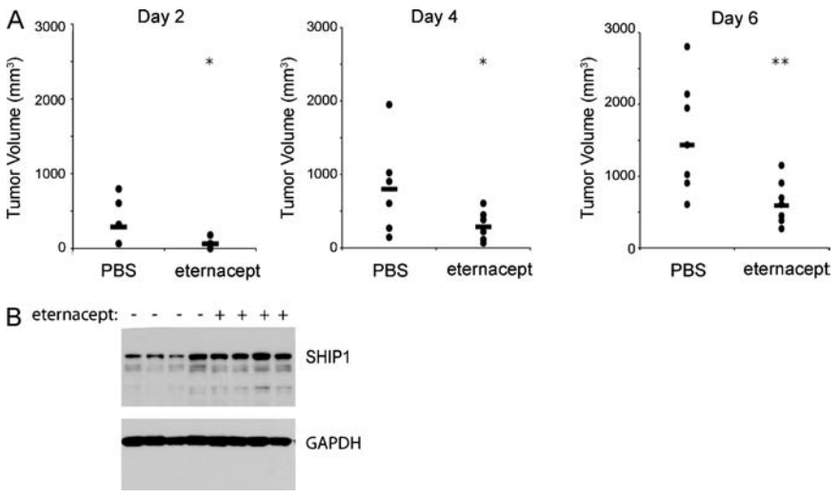

Figure 4. Anti-TNFα regimen inhibits DLBCL growth *in vivo*.

A. Sub-lethally irradiated (4 Gy) NOD/SCID mice were injected subcutaneously with 10^7 non-GC-DLBCL cells (Toledo). Upon establishment of palpable tumours, mice were injected every three days with 100 mg eternacept i.v., and tumour size was measured every second day with callipers ($n = 8$/group; $^{*}p < 0.03$; $^{**}p < 0.01$).

B. Xenograft tumours were excised from four mice in each group and lysates were analysed by Western blot using SHIP1 and GAPDH antibodies.

The paper explained

PROBLEM:

Diffuse large B-cell lymphoma (DLBCL) is clinically, morphologically and genetically a heterogeneous group of lymphomas involving malignant proliferation of large lymphoid B cells. DLBCL accounts for approximately 40% of adult non-Hodgkin lymphomas (NHL). Two prognostically different subgroups have been identified, with distinct gene expression profiles characteristic of either normal germinal centre (GC) B cells, or activated memory B cells with the GC B cell-like subgroup being correlated with a significantly better prognosis. Recent work has revealed unique miR signatures in each sub-group, but the relation of these to tumour growth and malignancy and their value as prognostic indicators has not been fully clarified.

RESULTS:

The authors identify the inositol phosphatase SHIP1 as a bonafide target of the oncogenic miR-155. They demonstrate that DLBCL cells display elevated levels of miR-155 and consequently diminished expression of SHIP1. They establish that both features are the result of autocrine stimulation by the pro-inflammatory cytokine TNF and go on to show that an anti-TNF regimen involving treatment with eternacept or infliximab is sufficient to reduce miR-155 levels and restore SHIP1 expression. These changes are accompanied by a reduction in cell proliferation and a substantial decrease in tumour burden in DLBCL xenografts.

IMPACT:

These findings strongly support the concept that cytokine-regulated miRs can function as a crucial link between inflammation and cancer, and illustrate the feasibility of anti-TNFα therapy as a novel and immediately accessible (co)treatment for DLBCL.

albeit no oncogenetically relevant target had been identified. Under physiological conditions, miR-155 plays an important role in the formation of GCs through the regulation of cytokine production (Thai et al, 2007). miR-155-deficiency was found to be associated with decreased TNFα production, a cytokine required for GC formation (Endres et al, 1999), whereas forced expression of miR-155 leads to elevated TNFα levels (Tili et al, 2007). As miR-155 is a TNFα-inducible transcript, these findings taken together suggest the possibility of a positive amplification loop. Strikingly, aberrant miR-155 expression in non-GC-DLBCL appears to be a consequence of an autocrine stimulation by TNFα rather than the result of a chromosomal translocation, as miR-155 and SHIP1 levels can be reduced or increased, respectively, through the administration of neutralizing anti-TNFα antibodies or soluble TNFα receptor. It is apparent that miR-155 is not the only target gene upregulated by TNFα, nor is it likely that SHIP1 is the sole target of miR-155. However, there is no doubt about the oncogenic properties of miR-155, or the tumour-suppressive effects of the inositol-phosphatases SHIP1 and PTEN. Extensive additional studies employing transgenic re-introduction of SHIP1 into miR-155-transgenic mice will be required to address the importance of SHIP suppression in the oncogenic transformation elicited by miR-155. Our current results nevertheless not only clearly illustrate that the expression levels of SHIP1 and miR-155 are valuable prognostic indicators in DLBCL, but also strongly support the concept that inflammatory miRs contribute to the development or progression of cancer. Most importantly, our studies demonstrate the feasibility of anti-TNFα therapy as a novel and immediately accessible (co)treatment for DLBCL.

MATERIALS AND METHODS

Cell culture

OCILY-3, OLILY-10 and Toledo cells were maintained in complete Iscove's DMEM (Dulbecco's modied Eagle's medium) + 20% human serum + 100 mg/ml penicillin/streptomycin + 2 mM L-glutamine (Invitrogen, Carlsbad, CA). SUDHL-4 and Daudi cells were maintained in complete Roswell Park Memorial Institute (RPMI) medium + 10% foetal calf serum (FCS) + 100 mg/ml penicillin/ streptomycin + 2 mM L-glutamine. Where indicated, cells were treated with 400 nM c-Jun N-terminal kinase (JNK) Inhibitor II (Calbiochem, San Diego, CA), and/or 20 ng/ml of human TNFα (Peprotech Inc., Rocky Hill, NJ). Cell proliferation was determined using a Beckman Coulter Counter.

Patient samples

Sections of frozen biopsies were stained for CD10 (clone: 56C6, Novocastra), Bcl-6 (clone: PGBGp, DAKO) and multiple myeloma oncogene 1 (MUM1) (MUM1p, DAKO) in a Techmate 500 Immunostainer using DAKO Envision K5007 as a secondary antibody. CD10 positive or Bcl-6 positive, MUM1 negative samples were classified as GCB-DLBCLas described by Hans et al (Hans et al, 2004). For LCD, lymph node sections were stained with haematoxylin and eosin (H&E) staining kit (Molecular Machines Industries, Glattbrugg, Switzerland), and lymphoma cells (1,000 cells/patient specimen) were isolated using the MMI CellCut (MMI). All human material was handled in full compliance with NIH guidelines and IRB approval.

Luciferase Assays

The putative target site of the miR-155 seed sequence in the 3′-UTR of hSHIP1 (WT; 5′-AGCTTGGGCTTCTTAATGCTTTCACCCCTCA-3′ and 5′-CTAGTGAGGGGTGAAAGCATTAAGAAGCCCA-3′) or a mutated variant (Mut; 5′-AGCTTGGGCTTCTGGGTGCTTTCACCCCTCA-3′ and 5′-

CTAGTGAGGGGTGAAAGCACCCAGAAGCCCA-3′) were cloned into the pmiReport firefly-luciferase vector (Applied Biosystems, Foster City, CA). 293 T cells at 50% confluency were transfected in triplicate using lipofectamin with the pmiReport luciferase vectors and either miR-CTL#1, miR-155 or a miR-155 variant (5′-GGGAUAGUGCUAAUC-GUGGGU; all from Dharmacon, Lafayette, CO) complementary to the altered putative target site introduced in the pmiReport luciferase vector. Renilla luciferase under the control of a cytomegalovirus (CMV) promoter was co-transfected to serve as internal reference. After 24 h, cells were washed in PBS and luciferase activities were measured using Promega's Dual Luciferase Assay System.

Transfection of miRs

OptiMem (Invitrogen) and Mirus transfection reagent (Mirus Bio/Fisher Scientific) were combined according to the manufacturer's instructions, and miR mimics or anti-miRs (mimics from Dharmacon; anti-miRs from Applied Biosystems) were introduced prior to addition of the transfection mix to the cells.

RNA isolation and quantitative polymerase chain reaction (qPCR)

RNA was extracted using TriZol following the manufacturer's protocol (Invitrogen), and miR kits (RT and TaqMan q-PCR primers, Applied Biosystems) were used for qPCR analysis of miR-155, U6 and U43 according to the manufacturer's instructions. qPCR analysis of SHIP1 and GAPDH mRNA was performed using specific primers for SHIP1 (SABiosciences, Frederick, MD) and GAPDH (Dharmacon). Relative expression was calculated using the comparative threshold cycle (Ct) method.

Western blot analysis

Cell lysates were subject to Western blot analysis using rabbit monoclonal antibodies against SHIP1, SHIP2 or GAPDH (Cell Signaling Tech, Danbars, MA), and blots were developed using ECL (GE Healthcare).

Xenografts

Nod/SCID mice were subcutaneously inoculated with 10^7 Toledo cells. Upon establishment of palpable tumours, the animals received either 100 μg eternacept or an equal volume of solvent intravenously every three days. Tumour size was measured after 2, 4 and 6 days. Tumours were excised at day 6, lysed and analysed by Western blot for SHIP1 expression. All experiments were approved by UCSD Institutional Animal Care Committee.

Author contributions

IMP performed the SHIP and miR-155 expression and function studies, and is responsible for the design and analysis of the experiments; DO performed the xenograft studies; EK created the luciferase constructs; AVM is responsible for the experiments using SHIP and PTEN-deficient mice; CH performed the LCMD and qPCR analysis on the biopsies; ER assisted in the collection of the clinical material; KG performed the mutation and promoter methylation analysis; RCR provided expert advise and guidance throughout this study and contributed to the writing of the manuscript; MD guided and coordinated the studies, supervised the design and analysis of the experiments and wrote the manuscript.

Acknowledgements

We thank Dr. Lassos, Miami, FL for kindly providing the OCILY-3, OLILY-10 and SUDHL-4 cell lines. This work was supported by NIH T32 CA09523 and K01 CA122192 grants to IMP, NIH R01 HL088686 grants to RCR, Novo Nordisk Foundation grants to KG and by NIH R01CA135531 grants to RCR and MD.

Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

Lymphoma Research Foundation:
http://www.lymphoma.org

Oncomine – Cancer profiling database:
http://www.oncomine.org

References

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, *et al* (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403: 503-511

Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857-866

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, *et al* (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 99: 15524-15529

Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, *et al* (2004) MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci USA 101: 11755-11760

Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, *et al* (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102: 13944-13949

Coiffier B (2001) Diffuse large cell lymphoma. Curr Opin Oncol 13: 325-334

Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, Croce CM (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci USA 103: 7024-7029

Endres R, Alimzhanov MB, Plitz T, Futterer A, Kosco-Vilbois MH, Nedospasov SA, Rajewsky K, Pfeffer K (1999) Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. J Exp Med 189: 159-168

Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269

Fruman DA (2004) Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. Curr Opin Immunol 16: 314-320

Habermann TM, Weller EA, Morrison VA, Gascoyne RD, Cassileth PA, Cohn JB, Dakhil SR, Woda B, Fisher RI, Peterson BA, *et al* (2006) Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. J Clin Oncol 24: 3121-3127

Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, *et al* (2004) Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103: 275-282

He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, *et al* (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828-833

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ (2005) RAS is regulated by the let-7 microRNA family. Cell 120: 635-647

Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, Cattan H, Enver T, Mager R, Boultwood J, *et al* (2007) MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer 121: 1156-1161

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, *et al* (2005) MicroRNA expression profiles classify human cancers. Nature 435: 834-838

Malumbres R, Sarosiek KA, Cubedo E, Ruiz JW, Jiang X, Gascoyne RD, Tibshirani R, Lossos IS (2009) Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. Blood 113: 3754-3764

O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D (2007) MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci USA 104: 1604-1609

Pasparakis M, Alexopoulou L, Episkopou V, Kollias G (1996) Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med 184: 1397-1411

Pedersen LM, Jurgensen GW, Johnsen HE (2005) Serum levels of inflammatory cytokines at diagnosis correlate to the bcl-6 and CD10 defined germinal centre (GC) phenotype and bcl-2 expression in patients with diffuse large B-cell lymphoma. Br J Haematol 128: 813-819

Pfreundschuh M, Ho AD, Cavallin-Stahl E, Wolf M, Pettengell R, Vasova I, Belch A, Walewski J, Zinzani PL, Mingrone W, *et al* (2008) Prognostic significance of maximum tumour (bulk) diameter in young patients with good-prognosis diffuse large-B-cell lymphoma treated with CHOP-like chemotherapy with or without rituximab: an exploratory analysis of the MabThera International Trial Group (MInT) study. Lancet Oncol 9: 435-444

Rai D, Karanti S, Jung I, Dahia PL, Aguiar RC (2008) Coordinated expression of microRNA-155 and predicted target genes in diffuse large B-cell lymphoma. Cancer Genet Cytogenet 181: 8-15

Roldo C, Missiaglia E, Hagan JP, Falconi M, Capelli P, Bersani S, Calin GA, Volinia S, Liu CG, Scarpa A, *et al* (2006) MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol 24: 4677-4684

Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Giltnane JM, *et al* (2002) The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 346: 1937-1947

Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, Gaasenbeek M, Angelo M, Reich M, Pinkus GS, *et al* (2002) Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med 8: 68-74

Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, Murphy A, Frendewey D, Valenzuela D, Kutok JL, *et al* (2007) Regulation of the germinal center response by microRNA-155. Science 316: 604-608

Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA, *et al* (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179: 5082-5089

